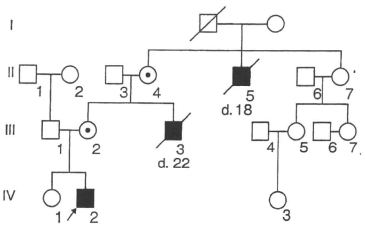

図2　Duchenne 型筋ジストロフィーの例—家系図

おいて，正確な診断は必須事項である．臨床診断が異なると当然ながら遺伝子検査によって変異が見出されず，また遺伝形式も異なるため，遺伝カウンセリングは誤った方向へ行ってしまう．

DMD では，家系図の分析，病歴，臨床所見（筋力低下，筋萎縮，筋肥大，徒手筋力テスト，深部腱反射など），血清 CK（creatine kinase）値によって，臨床的に診断が可能である．家系内の罹患者が死亡している場合や移動が困難である場合には，全身が分かる写真も有用である．家系内の罹患者における遺伝子検査がすでに行われ，確定診断の情報が得られる場合も増えてきており，発端者やその両親へのインフォームドコンセントの下に，遺伝子解析結果などの医学情報の提供を担当医に依頼することもある．

図2の例では，かかりつけ医からの診療情報と診察所見から，患児（IV-2）は DMD と臨床診断した．本例のように家系図から患児の母親が保因者と確定される場合もあり，患児の診断が，その母親の保因者診断となる場合も少なくない．このことが，遺伝子検査における検査前の十分な遺伝カウンセリングの必要性の理由でもある．

2. 遺伝形式の診断—家系図の作成

家系図の作成は遺伝性疾患の診療の基本である[3]．できるだけ詳しく，3世代くらいは遡って情報を得ることが望ましい．家系図を分析することにより遺伝形式が分かり，疾患の診断がなされたり，否定されたりする．

たとえば，各世代の男女に同様の疾患の患者が認められるとき，常染色体性劣性遺伝は否定される．また，母親を介して疾患が遺伝していることが考えられるとき，X 連鎖性疾患やミトコンドリア病を考える．父と息子が同様の疾患であるとき，X 連鎖性の疾患は否定される．図2からは II-4，III-2 が保因者であることは確定的であるが，III-5 は保因者であるか否か家系図からは判定できない．

3. インフォームドコンセント

文書により，疾患に関する医学的・医療的情報，遺伝形式，遺伝子検査の目的，検査の有用性と限界などについて十分な情報を提供し，遺伝子診断の説明を行う．被検者または代諾者（小児では主に両親）が DMD について十分に理解していない可能性もあるため，DMD に関する説明や医療の進歩の現状についての情報提供は重要である．

また，DMD の遺伝子検査では，ジストロフィン遺伝子のエクソンレベルの欠失や重複を示す家系においては，健康保険の適用となる遺伝子診断の精度は高く，迅速な診断ができるが，点変異を含む微小変異の症例も約3割に認められ，採血による遺伝子検査には限界もある．その場合には筋生検を行い，骨格筋由来の mRNA から cDNA を合成し，塩基配列の決定を行う．これらの話し合いの後に，被検者または代諾者の自己決定の下で同意書に署名を得る．

4. スタッフ・カンファランス

以上のような臨床情報を基に，図1のように，スタッフが話し合い，情報を共有し，今後の方針を考察する．

5. 遺伝子検査

図2のケースでは，multiplex ligation-dependent probe amplification（MLPA）法を実施し，コントロールと比較をする．患児はエクソン 41，42，43 の欠失を示し（図3），DMD の確定

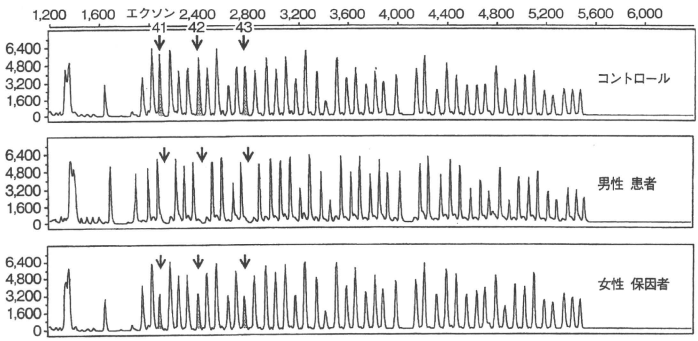

図3　Duchenne 型筋ジストロフィーの例— MLPA 法

診断となった.

6. 遺伝子検査後のフォローアップ

以上のように,遺伝カウンセリングにおいて,患者とその家族のニーズにある程度応えることができても,遺伝性疾患は治療不可能なものが多い.最新の遺伝子研究や治療研究の進歩に関する情報を提供するとともに,その家系における遺伝医学的な問題に応えることも必要である.

本家系では,母の従妹である III-5 が保因者診断を希望した.図3の MLPA 法により保因者診断も可能である.保因者である場合は図3の下段のようにエクソン 41, 42, 43 が非保因者の 1/2 量となる.保因者であると診断されたケースにおいては,その女性の妊娠において,遺伝カウンセリングを通して,できる限りの援助を行うことを話し[4],疾患をもって生まれた場合の子どものケア,サポート体制についても説明する.患者会などの情報も役立つ.また,出生前診断に関するガイドラインについて解説し,絨毛穿刺,羊水穿刺による遺伝子検査,受精卵診断としての着床前遺伝子検査について解説し,その女性の自己決定をサポートしていくことが重要である.

おわりに

遺伝子検査においては,疾患に関する医学的情報,遺伝形式,検査の目的,検査の有用性と限界などについて十分な説明を行い,理解を得たうえで,その意思を尊重することが重要である.さらに,遺伝子検査の結果は本人のみならず,家族・血縁者にも関係する.必要に応じて臨床遺伝専門医や認定遺伝カウンセラーなどの遺伝医学の専門家への紹介を行い,遺伝カウンセリングの機会を提供することが望まれる.

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検査時期による遺伝学的検査と問題点

保因者診断と遺伝カウンセリング

Carrier diagnosis and genetic counseling

斎藤加代子

Key words：家系図，保因者，遺伝子検査，遺伝カウンセリング，臨床遺伝専門医

はじめに

近年の遺伝医学分野における目覚ましい進歩により，ヒトゲノムの解析が進み，疾患の遺伝子が同定され，疾患の発症機序が明らかになるとともに，多くの遺伝性疾患の遺伝子診断が可能になった．また，遺伝子変異の状態により臨床的な重症度の判定を下し，適切な治療や療育の方針を立てることに貢献ができるようにもなりつつある．遺伝子診断は，疾患の確定診断，発症前診断，保因者診断，出生前診断などに利用される．疾患の確定診断を行い，明確な診断がつくことによって，治療につながる疾患も出てきた．例えば，小児大脳型副腎白質ジストロフィーにおいて発症前や発症後早期の造血幹細胞移植による症状の改善や進行の停止が期待でき，早期に診断を確定することが有効である．一方，いまだ治療法がない疾患の確定診断，発症前診断，保因者診断，出生前診断において，様々な倫理的社会的問題が生じうる．したがって，遺伝子診断は一般の臨床検査と同格には扱えず，遺伝カウンセリングの中の一つの行為として位置付けられる．

保因者であるか否かは，疾患によっては家系図，診察，生化学検査，病理組織学的検査でわかることもあるが，遺伝子検査による保因者診断によって正確な判定ができるようになってきた．重篤な疾患を有する児を出産した両親，X連鎖性の難治性疾患の患児の血縁の女性は自分がその疾患の遺伝子変異を有するか，自分の子はその疾患に罹患するか悩む．このようなクライエントに対して，正確な情報提供，保因者診断を受けるか否かの意思決定におけるサポート，遺伝子検査の実施，検査結果の開示とその後のケアを含むフォローアップのシステムが保因者診断における遺伝カウンセリングである．

1. 保因者診断

a. 家系図からの保因者診断

家系図の聴取は遺伝子医療において非常に重要である[1]．図1の副腎白質ジストロフィーの例のように，男性患者の母親で，その男性同胞(兄弟)に罹患者がいる場合など，家族歴から確実に保因者としか考えられない場合を絶対的保因者(obligate carrier)という．時に，X連鎖劣性遺伝病の保因者で，症状を呈することがあり，manifesting carrierという．Duchenne型筋ジストロフィー(DMD)では患者の母親や姉妹が筋症状を呈することがある．

b. 遺伝子検査による保因者診断

遺伝子医療の現場において，遺伝子検査の目的の多くは患者本人の確定診断である．患者本

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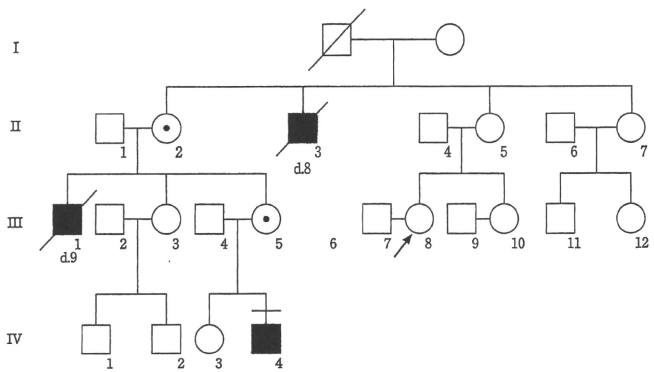


図1 副腎白質ジストロフィーの家系図

III-8: クライエント25歳．従姉から遺伝学的検査を勧められて来院．
II-3: 小学校低学年で発症し，8歳で死亡．
III-1: 6歳時ALDを発症．9歳にて死亡．
IV-4: 現在9歳．8歳で発症し，骨髄移植を受けている．遺伝子変異同定．

人の遺伝子変異が同定されると，遺伝子情報をもとに，その家系の血縁者が患者と同じ遺伝子変異を有しているか，すなわち保因者診断，発症前診断，更には出生前診断の対象となりうる．これらは患者本人の確定診断のための遺伝子検査に比して，被検者は診断の時点では症状のない未発症者または発症しているか不明な胎児を対象としたものである．いずれも被検者自身，あるいは被検者の子孫における将来の発症を予測する，または回避することを目的として行われる．疾患についての医学的な情報，遺伝に関する情報などの説明を含めて，十分な遺伝カウンセリングと心理的社会的支援が行えるなど，より専門性の高い体制のもとで対応すべき問題である．遺伝カウンセリングを含む遺伝子医療を実施している本学遺伝子医療センターにおいて，遺伝子検査の申し込みを受けた内訳を図2に示す．保因者診断はDMDが最も多かったが，常染色体劣性遺伝性疾患である福山型筋ジストロフィーや脊髄性筋萎縮症でも申し込みがあった．DMDにおける保因者診断は先に述べた家系図による診断のほかに，①血清creatine kinase(CK)値の高値，②生検筋における抗ジストロフィン抗体を用いた免疫組織化学染色(図3)におけるモザイク状の陰性筋細胞の存在，③遺伝子検査がある．ここでは遺伝子検査の実例を示す(図4)．

1) 定量的PCR法(gene dose測定法)

PCR法により増幅した産物をgene scanによって定量する方法である．内部コントロールと面積の比較によって検討をする．保因者である場合は発端者における欠失exonのgene doseは，非保因者の1/2量となる．図4-aに示す症例では，II-3が示していたジストロフィン遺伝子exon 53，54の欠失を，II-4の女性は示していないことから，保因者ではないと結論できる．

2) Multiplex ligation-dependent probe amplification(MLPA)法[2]

DMDの79個のexonを同時に増幅し遺伝子欠失，重複を面積で比較評価できる方法である．図4-bに示すように罹患男性はexon 41，42，43の遺伝子欠失を示し，その家系の女性において検査したところ，コントロールの1/2の遺伝子量を示したため，保因者であると判定した．

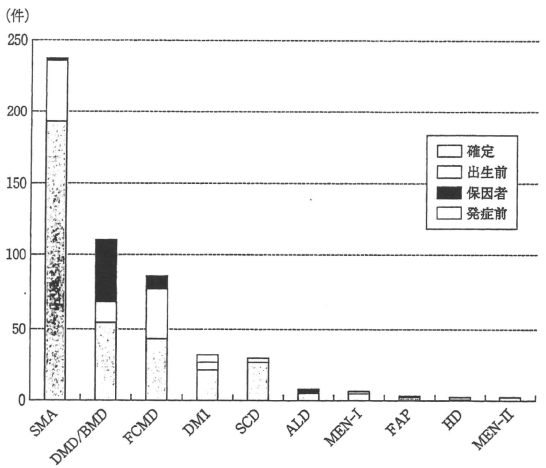


図2　東京女子医科大学遺伝子医療センターにおける遺伝子検査の申し込みの内訳(2004.5.1–2008.4.30まで)(n=516件)

SMA: 脊髄性筋萎縮症，DMD: Duchenne型筋ジストロフィー，BMD: Becker型筋ジストロフィー，FCMD: 福山型筋ジストロフィー，DM1: 筋強直性ジストロフィー，SCD: 脊髄小脳変性症，ALD: 副腎白質ジストロフィー，MEN–I: 多発性内分泌腺腫瘍症1型，FAP: 家族性ポリポーシス，HD: Huntington病，MEN–II: 多発性内分泌腺腫瘍症2型．

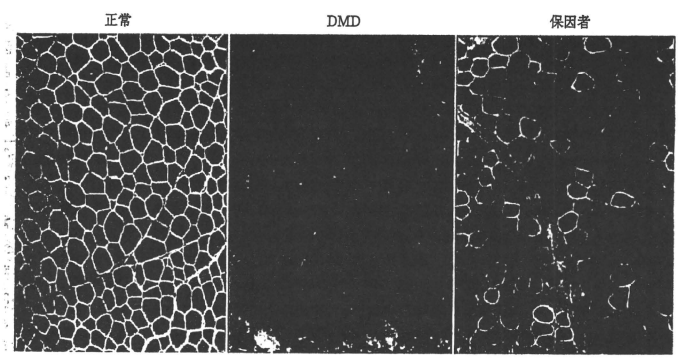
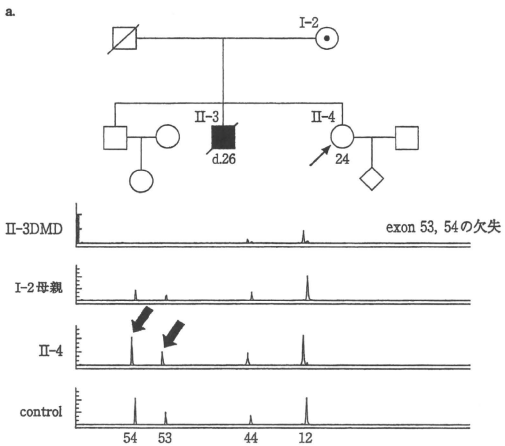


図3　生検筋組織における抗ジストロフィン抗体を用いた免疫組織化学染色



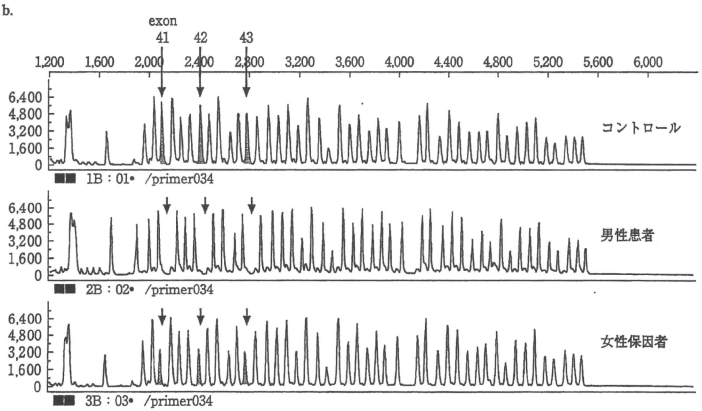
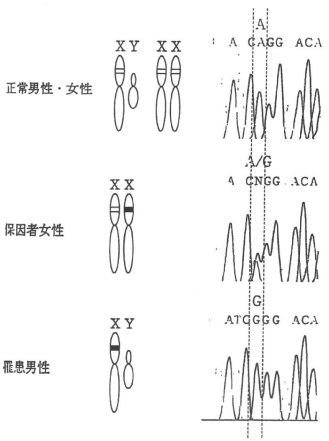


図4 DMDにおける遺伝子検査による保因者診断

a: 定量的PCR法(gene dose測定法)

b: multiplex ligation-dependent probe amplification(MLPA)

c: 塩基配列決定法



また，脊髄性筋萎縮症の患児の血縁者の保因者診断においてもMLPA法は有効である．

3）塩基配列決定法

血液または筋組織からmRNAを調製後，cDNAとして，ジストロフィン遺伝子の全領域をカバーするプライマーを設計して，RT-PCR法によって増幅し，塩基配列決定(シーケンス)する．図4-cでは，正常ではA(アデニン)であるところが，罹患男性ではG(グアニン)となっていた．その家系の女性において，AとGの両者が認められ，保因者であると判定した．

2. 保因者診断における遺伝カウンセリング

保因者診断は，遺伝性疾患において家系内に常染色体劣性遺伝病やX連鎖劣性遺伝病の患者がいる場合，当事者が保因者であるかどうかを明らかにして，将来，子孫が同じ遺伝病に罹患する可能性を予測する目的でなされる．すなわち，DMDのmanifesting carrierのように症状を有する場合は，本人の確定診断による健康管理の目的を有するが，多くの保因者診断は，被検者に対して本人の健康管理の情報というより，子孫の遺伝性疾患の罹患の予測の情報を得るために行われるものである．保因者診断によって被検者が保因者であると確定した場合，被検者が妊娠したときに自身の子がその疾患に罹患しているか否か出生前診断を希望しうる．したがって，対象となる疾患が出生前診断の適応となるのか，十分な考慮が必要となる．このことを十分に説明し，理解を得ることを保因者診断の遺伝カウンセリングにおいて心がける必要がある．将来の自由意思の保護という観点から，小児に対する保因者診断は基本的に行われるべきではない．

「遺伝カウンセリング」について，UNESCOの「ヒト遺伝情報に関する世界宣言(2003)」[5]第11条では「健康に関わる重要な意味を持つ可能性がある遺伝学的検査を行おうとする場合，当事者が遺伝カウンセリングを適切な方法で受けられるようにすべきである．遺伝カウンセリングは非指示的であり，文化的に適合したものであり，かつ当事者の最大の利益と一致したものであるべきである．」と述べている．

遺伝カウンセリングでは，①当事者が情報に基づいて決定できるように，個人やカップルに対し，選択肢や医学知識について理解を深めるために援助し，②当事者がよく理解したうえで，その遺伝的問題に対処していくように援助する．また，③罪の意識を取り除き，④個人やカップルが親となることへの目標に到達できるように援助する．遺伝性疾患の遺伝カウンセリングに当たる者としては，疾患に対する正しい知識と情報を入手し，患者とその家族の遺伝に関する疑問に正確に答えることが必要である．

遺伝医学関連学会による「遺伝学的検査に関するガイドライン(2003.5)」[6]でも「遺伝学的検査は臨床的および遺伝医学的に有用と考えられる場合に考慮され，総合的な臨床遺伝医療の中で行われるべきである．遺伝学的検査を行う医療機関においては，遺伝カウンセリングを含めた総合的な臨床遺伝医療を行う体制が用意されていなければならない」としている．すなわち，遺伝学的検査は，十分な遺伝カウンセリングを行った後に実施することが必要である．遺伝カウンセリングは，臨床遺伝学の専門的知識・経

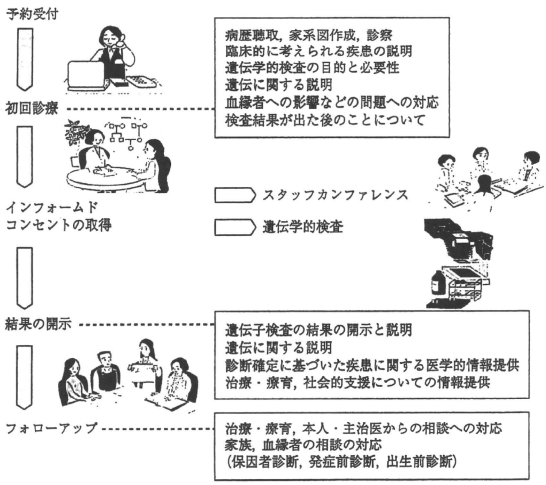


図5　遺伝カウンセリングの流れ

験をもち，遺伝カウンセリングに習熟した臨床遺伝専門医(日本人類遺伝学会と日本遺伝カウンセリング学会の共同認定の専門医)などにより被検者の心理状態を常に把握しながら行われるべきである．遺伝カウンセリング担当者は，本人および家族などの心理社会的支援を行うために，臨床心理専門職，遺伝看護師，ソーシャルワーカーなどと協力をして，チーム医療として実施することが望ましい(図5)．遺伝子の中には個人の生命現象のほとんどすべての情報が含まれており，遺伝子情報には厳しい守秘義務が付随する．また，遺伝性疾患に関する知識の普及は他の疾患に比べて低い．遺伝カウンセリングに当たる者は，疾患の頻度，疾患の自然歴，再発危険性(遺伝的予後)，遺伝子診断のもつ意義などについて正確で新しい知識を有し，その内容を本人と家族に理解できる言葉で説明することが求められる．そして，それぞれの家族のニーズに対応する関連情報を提供し，本人や家族がその内容を理解したうえで意思決定できるように，高い倫理観をもってサポートすることが大切である．

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Original article

Comprehensive genetic analyses of *PLP1* in patients with Pelizaeus–Merzbacher disease applied by array-CGH and fiber-FISH analyses identified new mutations and variable sizes of duplications

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Abstract

Pelizaeus–Merzbacher disease (PMD; MIM#312080) is a rare X-linked recessive neurodegenerative disorder. The main cause of PMD is alterations in the proteolipid protein 1 gene (*PLP1*) on chromosome Xq22.2. Duplications and point mutations of *PLP1* have been found in 70% and 10–25% of all patients with PMD, respectively, with a wide clinical spectrum. Since the underlining genomic abnormalities are heterogeneous in patients with PMD, clarification of the genotype-phenotype correlation is the object of this study. Comprehensive genetic analyses using microarray-based comparative genomic hybridization (aCGH) analysis and genomic sequencing were applied to fifteen unrelated male patients with a clinical diagnosis of PMD. Duplicated regions were further analyzed by fiber-fluorescence *in situ* hybridization (FISH) analysis. Four novel and one known nucleotide alterations were identified in five patients. Five microduplications including *PLP1* were identified by aCGH analysis with the sizes ranging from

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374 to 951-kb. The directions of five *PLP1* duplications were further investigated by fiber-FISH analysis, and all showed tandem duplications. The common manifestations of the disease in patients with *PLP1* mutations or duplications in this study were nystagmus in early infancy, dysmyelination revealed by magnetic resonance imaging (MRI), and auditory brain response abnormalities. Although the grades of dysmyelination estimated by MRI findings were well correlated to the clinical phenotypes of the patients, there is no correlation between the size of the duplications and the phenotypic severity.

Keywords: Array-based comparative genomic hybridization (aCGH); Fiber-FISH; Fluorescence *in situ* hybridization (FISH); Pelizaeus–Merzbacher disease (PMD); Proteolipid protein 1 (*PLP1*)

1. Introduction

Pelizaeus–Merzbacher disease (PMD; MIM#312080) is a rare X-linked recessive neurodegenerative disorder characterized by early onset nystagmus and hypotonia later evolving into spastic tetraparesis, dystonia, ataxia, and developmental delay usually beginning in the first year [1–3]. The main cause of PMD is alterations in the proteolipid protein 1 gene (*PLP1*; MIM#300401) on chromosome Xq22.2 [4–6], which encodes 2 proteins, PLP1 and the splicing variant, DM20, both of which are abundantly expressed in oligodendrocytes [3]. PLP1 is thought to play a major role in myelin sheath formation by promoting sheath compaction [7]. Within the heterogeneous group of dysmyelinating disorders, PMD accounts for 6.5% of all cases [8].

It has been proposed that patients with *PLP1*-related inherited dysmyelinating disorders should be clinically divided into 3 subgroups in order of decreasing severity: connatal, classic, and X-linked spastic paraplegia type 2 (SPG2; MIM#312920) [9]. Duplications of *PLP1* can be found in up to 70% of all patients with PMD, indicating that increased *PLP1* dosage is deleterious for normal myelination [10,11]. Point mutations in *PLP1* have been found in 10–25% of PMD cases with the entire clinical spectrum [11], ranging from the most severe connatal form to the least severe SPG2 form, depending on the affected domain of the protein [9]. Although there are characteristic clinical and radiological features of PMD [1,12], molecular and/or cytogenetic analyses are necessary for final diagnosis because *PLP1* is only expressed in the central nervous system and there are no practical biochemical tests available. The first step in genetic testing should be a genomic dosage analysis of *PLP1* because the major genetic aberration is duplication of *PLP1*. For this purpose, various methods have been used, including southern blotting [6], quantitative polymerase chain reaction (PCR) [13], fluorescence *in situ* hybridization (FISH) [14], multiplex ligation-dependent probe amplification (MLPA) [15], and multiplex amplifiable probe hybridization (MAPH) [16]. Recently, microarray-based comparative genome hybridization (aCGH) has emerged as a novel technology that enables detection and determination of the size of the duplicated or deleted genomic intervals [17]. In case of normal dosage of *PLP1*, nucleotide sequences of *PLP1* should be examined [18]. Here, we report our recent studies to develop comprehensive molecular and cytogenetic analyses to diagnose patients with PMD and to understand the pathogenic mechanism of PMD and its correlation between clinical phenotypes.

2. Materials

Fifteen unrelated male patients (age span from 1 year to 20 years old) with congenital dysmyelination were referred to us for genetic diagnosis based on the clinical diagnosis as PMD. Clinical information and radiographic findings by magnetic resonance imaging (MRI) for the patients were obtained from attending doctors. Based on the approval by the ethics committee at the institution, informed consents were obtained from patient's families, and peripheral blood samples were obtained from all patients. Lymphoblast cell lines were established from lymphocytes extracted from peripheral blood samples by immortalization with Epstein-Barr virus. Three of the fifteen patients (P1, P3, P4) had been diagnosed as having *PLP1* duplications by previously performed comparative PCR amplification method (data not shown).

Genomic DNAs of the patients were extracted from peripheral blood samples using the QIAquick DNA Extraction Kit (QIAGEN, Hamburg, Germany). Metaphase or prometaphase chromosomes were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes or lymphocyte cell lines according to standard techniques.

One extra cell line (S1) that showed duplication of Xp22.31 including steryl-sulfatase precursor gene (*STS*) as determined by aCGH, was derived from a non-PMD mentally retarded patient and used for fiber-FISH analysis as a positive control of *STS* duplication.

Population-based control DNA samples were obtained from 100 healthy Japanese volunteers.

3. Methods

3.1. aCGH analysis

aCGH analysis, using the Human Genome CGH Microarray 105A chip (Agilent Technologies, Santa

Clara, CA), was performed according to the manufacturer's instructions [19]. The data was extracted with Feature Extraction version 9 (Agilent Technologies) and visualized by CGH Analytics version 3.5 (Agilent Technologies). Statistically significant aberrations were determined using the ADM-II algorithm in CGH Analytics version 3.5 (Agilent Technologies).

3.2. Fiber-FISH analysis

Phytohemagglutinin-stimulated lymphocytes or lymphoblasts were harvested using a routine procedure that generates metaphase chromosomes and interphase nuclei. The fiber-FISH slides were prepared as follows: approximately 20 μl of cell suspensions containing metaphase and prometaphase chromosomes were pipetted onto a slide that was then dipped into a 10% sodium dodecyl sulfate (SDS) solution and removed slowly. Bacterial artificial chromosome (BAC) clones were selected from an in-silico library (UCSC Human genome browser, March 2006); RP4-540A13 and RP5-1055C14 mapped to the region surrounding *PLP1*, CTD-2171N231 and RP11-98J1 mapped to the *STS* region of Xp22.31. DNAs from the BAC clones were extracted using GenePrepStar PI-80X (Kurabo, Osaka, Japan), and labeled with digoxigenin-11-dUTP or biotin-16-dUTP (Roche Applied Science, Mannheim, Germany) by nick translation and denatured at 70 °C for 5 min. After hardening process with incubation at 65 °C for 150 min, the chromosome slides were denatured in 70% formamide/2× standard saline citrate (SSC) at 70 °C for 2 min, and then dehydrated at −20 °C in ethanol. The probe-hybridization mixture was applied on the chromosome slides and incubated at 37 °C for more than 16 h. The slides were then washed in 50% formamide/2× SSC at 37 °C for 12 min, 2× SSC at room temperature for 10 min, 1× SSC for 10 min, and 4× SSC for 10 min. And finally, the slides were incubated with 1% bovine serum albumin (BSA), 4× SSC, Fluorescein anti-biotin (Vector, Burlingame, CA, USA) and Anti-digoxigenin-rhodamine, Fab fragments (Roche) at 37 °C for 1 h. Slides were washed 3 times: in 4× SSC for 5 min, in 0.05% Triton-X-100/4× SSC for 5 min with shaking, and finally in 4× SSC for 5 min. The slides were then mounted in antifade solution containing 4′,6-diamino-2-phenylindole (DAPI) stain. Photomicroscopy was performed using a LICA CTR6000 microscope containing a quad filter set with single band excitation filters (Leica Microsystems, Tokyo, Japan).

3.3. PLP1 mutation analysis

The sequence of the patients' 7 coding exons of *PLP1* was determined using the neighboring intronic primers reported by Hobson et al. [20] and a BigDye Terminator Cycle Sequencing kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). One hundred control samples were genotyped to verify that the *PLP1* mutation identified in the PMD patients was not found in the general population.

4. Results and discussion

4.1. Genetic diagnosis of PMD

Three distinct genetic mechanisms responsible for PMD have been reported: (1) Loss of PLP1 function caused by null mutations or deletions; (2) gain of toxic function (the PLP1 mutant protein accumulates in the endoplasmic reticulum, triggering increased oligodendrocyte cell death by apoptosis resulting in dysmyelination); (3) overexpression of PLP1 due to genomic duplication [3,7]. As mentioned in Section 2, three (P1, P3, and P4) of the 15 patients had been diagnosed as having *PLP1* duplications by previously performed comparative PCR amplification method, which were re-confirmed by aCGH and fiber-FISH in this study. Subsequently, two (P2 and P5) of the remaining twelve subjects were newly diagnosed as having *PLP1* duplications by aCGH. For the remaining ten patients without genomic duplications, we analyzed the genomic sequence of *PLP1* and identified three missense mutations, one splicing mutation, and one 3-bp deletion (Table 1). Thus, we were unable to determine genetic causes for the phenotype in the remaining five patients, and there is no patient who showed deletion of *PLP1*.

4.2. Detection of genomic duplications of PLP1 by aCGH

aCGH is a revolutionary platform that has been recently adopted in the clinical laboratory. The primary advantage of aCGH is that the array is capable of simultaneously detecting DNA copy changes at multiple loci over the whole genome [21].

In the present study, aCGH analysis identified gains of genomic copy numbers including *PLP1* in five subjects (P1, P2, P3, P4, P5), and the sizes of the chromosomal duplications were 374, 461, 676, 858, and 951-kb, respectively (Table 1 and Fig. 1a). The 461-kb duplication identified in P2 was not detected using standard FISH analysis at another medical facility previously, indicating the advantages of aCGH testing for PMD. There was no genomic copy number aberration in the remaining ten subjects (data not shown). A genomic copy number gain was identified on Xp22.31 with the size of 1.5-Mb in the sample from S1 (Fig. 1b).

4.3. Fiber-FISH analysis

aCGH holds the promise of being the initial diagnostic tool in the identification of visible and submicroscopic

Table 1
Clinical characteristics of the patients with PLP1 duplications or mutations.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Clinical subtype	Connatal	Classic	Connatal	Connatal	Connatal	Connatal	Connatal	Connatal	Connatal	Connatal
Age at examination	1 year	14 years	20 years	4 months	2 years	2 years	1 year	1 year	4 months	1 year 5 months
Disease onset	1 month	1 day	NA	1 day	1 month	2 months	1 day	1 week	1 month	1 month
Symptoms at onset	Nystagmus	Nystagmus	NA	Nystagmus	Nystagmus Hypotonia	Nystagmus	Asphyxia	Abnormal ABR	Microcephaly*	Nystagmus
Severity score	0	2	0	0	0	0	0	0	0	0
Family history	+	None	NA	None	None	None	None	None	None	None
Age at death				4 years						
Psychomotor development										
Head control	None	7 months	None	None	None	None	None	None	None	None
Sitting	None	4 years	None	None	None	None	None	None	None	None
Walking	None	4 years	None	None	None	None	None	None	None	None
Last evaluation	14 years	14 years	33 years	4 years	2 years	2 years	1 year	4 years	5 months	1 year 5 months
Neurological signs										
Nystagmus	+	+	None	+	+	+	+	+	+	+
Muscular hypotonia	+	None	+	+	+	+ (upper limbs)	+	+	+	+
Pyramidal signs	None	+	+		+	+ (lower limbs)	None	None	None	
Ataxia		+								
Tremor	+									
Seizures				+				+	+	Sensori-neural
deafness										
Other symptoms		Dysarthria		Choreoathetosis			Strider		Microcephaly	
Disease course		Deterioration; now only sitting								
ABR findings	Only I–III waves	Only I–III waves	NA	Only I wave	Only I wave	Only I wave	Only I–III waves	Only I wave	Only I wave	Only I–II waves
MRI findings	Delayed myelination	Incomplete myelination	NA	Hypomyelination	Hypomyelination	Delayed myelination	Delayed myelination	Delayed myelination	Delayed myelination	Hypomyelination
Genotype	Duplication	Duplication	Duplication	Duplication	Duplication	Missense mutation exon 2c.149A> G(p.Tyr50Cys)	Missense mutation exon 3c.247G> A(p.Gly83Arg)	Missense mutation exon 3c.254TA> Cp.Leu85Pro	Splicing mutation intron 3IVS3-1G>C (splicing error)	Nucleotide deletion exon 3c.238_240delTTC (p.Phe80del)
	374-kb	461-kb	676-kb	858-kb	951-kb					

*, His microcephaly is −3.2SD; NA, not available; MRI, magnetic resonance imaging; ABR, auditory brain response.

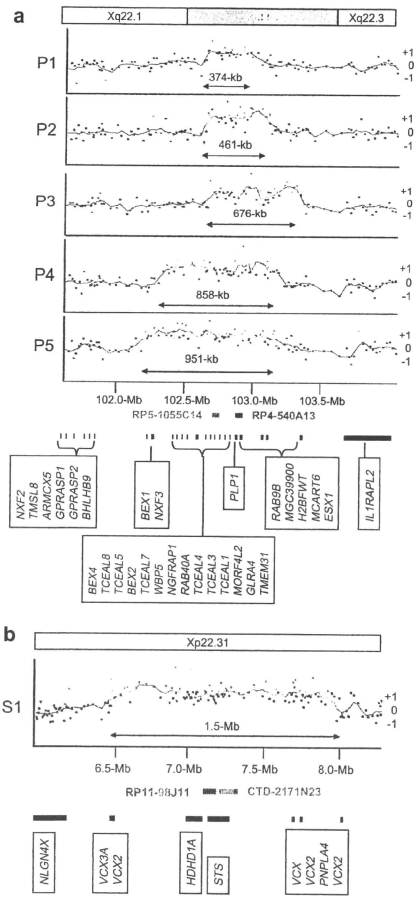

Fig. 1. Results of aCGH. CGH Analytics ver 3.5 (Agilent technologies) visualized genomic copy number aberration on Xq22.2 including *PLP1* (P1, P2, P3, P4, P5) (a) and on Xp22.31 including *STS* (S1) (b). The *X*-axis indicates physical position of chromosome X, and the scales of chromosome bands and physical position were depicted top and bottom, respectively. The *Y*-axis indicates the signal $\log_2$ ratio; positive and negative numbers indicate gain and loss of genomic copy numbers, respectively. The locations of the indicated genes (black rectangles), known copy number variations (CNVs) (orange rectangles), and the two BAC probes used in fiber-FISH analyses with green and red rectangles are shown under the figure depicted on the map according to the scale.

chromosome abnormalities [22], but it does not provide genome position or orientation information. Several cases have been reported in which the duplication is non-contiguous and the additional copy is found in a cytogenetically distinguishable band on the X chromosome (Xq22 and Xq26.3) [23]. Therefore, we should reconfirm the results of aCGH by another method including FISH analysis, especially in case of genetic counseling [23].

We checked the signals by conventional FISH analysis using metaphase, and translocations were denied in all samples (data not shown). Subsequently, two-color fiber-FISH analyses were performed to confirm the directions of the genomic duplications of *PLP1* in the five subjects, and the all duplicated segments were inserted in tandem (Fig. 2). The subjects with the longer duplicated regions (P4 and P5) showed longer intervals between the 2 sets of probe signals, which are consistent with the results of the aCGH analysis (Fig. 1a). The gain of genomic copy number on Xp22.31 in S1 was also analyzed by fiber-FISH, which showed inverted segments (Fig. 2).

Detection and visualization of *PLP1* duplications require specific molecular and cytogenetic technologies. Duplication of chromosomal regions can be determined by FISH analysis as a doublet signal in interphase chromosomes derived from immortalized lymphocyte cell lines [14,24]. However, when the duplicated region is very small and the locations of the duplicated segments are too close to each other, it is difficult to identify signals independently even if we use interphase chromosomes. In such cases, stretched chromatins rather than

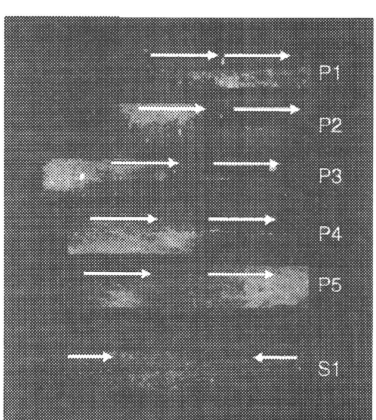

Fig. 2. The results of two-color fiber-FISH analyses. White arrows indicate the direction of duplicated segments.

the conventional interphase or metaphase chromosomes can be used for fiber-FISH analysis [25]. In this technique, two copies of the gene can be visualized as a doublet signal [14].

Interestingly, in PMD patients with *PLP1* duplications, the rearrangement breakpoints for each patient are different, yielding duplicated genomic segments of varying lengths [25–28]. Based on the genomic region around *PLP1*, Woodward et al. suggest that duplicated segments or low copy repeats (LCRs) may promote instability [26]. In this study, the distal ends of the duplicated segments were located in a copy number variation (CNV) region (cnp1417: 102,969,058–103,341,717) [29] in all five subjects, and the proximal ends were differently expanded (Fig. 1a). These findings were similar to the majority of the 11 duplications found by Woodward et al. [26]. Additional studies have shown that *PLP1* duplication events may be stimulated by LCRs or by nonhomologous pairs at both the proximal and distal breakpoints [21]. Despite the variation in size, the duplications encompassing *PLP1* are usually found in tandem [26,30]. All of our subjects with *PLP1* duplications had tandem duplications, as revealed by fiber-FISH analysis (Fig. 2). We also analyzed the region of microduplications of *STS* on Xp22.31, known as the CNV region, by fiver-FISH, which demonstrated that the duplicated segment was inserted in inverted direction (Fig. 2). This indicates that the chromosomal duplication mechanism varies depending on the location.

4.4. PLP1 mutations

To attempt to identify the genomic anomalies responsible for PMD in the remaining ten patients without *PLP1* duplications, we sequenced all the seven exons of *PLP1* and identified nucleotide alterations in five patients (Table 1, Fig. 3). Three of them were missense mutations, i.e. c.149A>G (p.Tyr50Cys) in exon 2, c.247G>A (p.Gly83Arg) in exon 3, and c.254T>C (p.Leu85Pro) in exon 3, in P6, P7, and P8, respectively. One was a splicing mutation, IVS3-1G>C in intron 3, in P9. Another was 3-bp deletion, c.238_240delCTT (p.Phe80del) in exon 3, in P10. Although c.149A>G was previously reported by Hübner et al. [18], the others were novel.

The two missense substitutions, c.247G>A and c.254T>C, are located within the second hydrophobic transmembrane domain. The nucleotide alteration, IVS3-1G>C, is located within the consensus splicing acceptor site. There is a similar known splicing mutation at the same splicing acceptor site, but it was IVS3-1G>T [31]. As PLP1 is expressed only in the central nervous system, we cannot confirm splicing alterations by RT-PCR. However, the mutations in the consensus splicing sites are believed to cause splicing abnormalities. The other novel 3-bp deletion, c.238_240delTTC, in exon 3 will cause in-frame amino acid deletion. All four novel

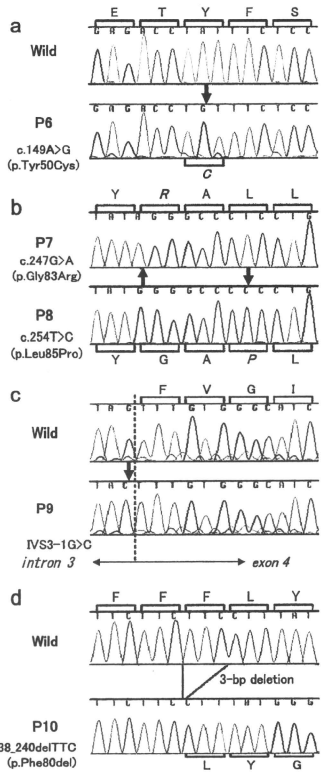

Fig. 3. Partial sequence electrophoregrams of *PLP1* mutations identified in this study. Thick arrows and italic amino acid symbols indicate the positions of mutations and altered amino acids, respectively. The broken line indicates exon–intron boundary (C). wild; sequence of wild type.

mutations were not detected in the 100 control samples, leading us to conclude that these should not be polymorphisms. Because it is well known that the genomic sequence of *PLP1* is highly conserved between species [32], these novel mutations should be pathogenic for patients with PMD. Previously, we reported two pathogenic *PLP1* mutations identified on the patients with the connatal form of PMD, and one of which, jimpy^{msd} mutation, was identical with the mice model [33,34]. Including them, more than 100 *PLP1* mutations have been reported to date (see the GeneTests Web site: http://www.genetests.org). Mutations

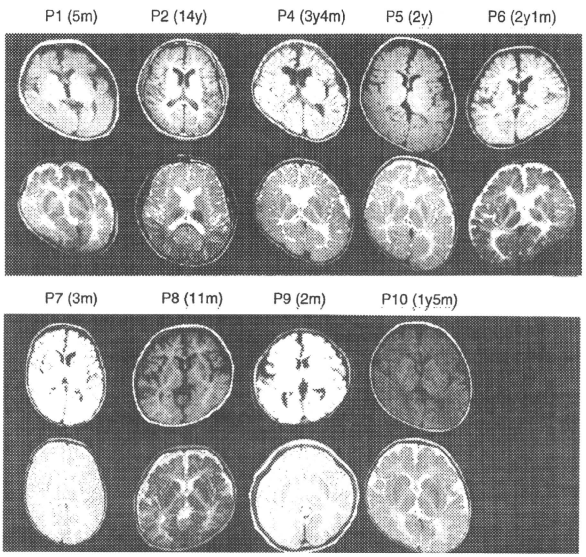

Fig. 4. Brain MRIs of nine patients analyzed in this study. Axial T1- and T2-weighted images are in the top and bottom rows, respectively. Ages at the time of examination are shown in brackets. y; years, m; months.

are distributed throughout all of the *PLP1* coding exons, and each mutation is usually unique to a family [7]. The fact that the majority of *PLP1* missense mutations cause more severe phenotypes than null mutations suggests that the profound dysmyelination resulting from *PLP1* point mutations probably arises not from the absence of functional protein, but rather from a cytotoxic effect of the mutant protein [3].

4.5. Correlation of PLP1 genotypes with PMD phenotypes

The clinical phenotypes of ten subjects who were diagnosed as having *PLP1* duplications (five) or *PLP1* nucleotide alterations (five) are summarized in Table 1. P2 is the only patient with the milder classic form of PMD, and demonstrated the greatest walking ability of all cases in this study. The other subjects were diagnosed with the connatal form of PMD with severe developmental delay. P4 died at age 4, although no details were provided.

Brain MRIs were obtained from nine patients and shown in Fig. 4. All of them showed abnormal intensity in the white matter. Although P2 showed normal high intensity in T1-weighted image (T1), that of the frontal lobe in T2-weighted image (T2) is higher than that of the occipital. This indicated incomplete myelination. P6, P7, P8, and P9 showed high intensity in both T1 and T2, indicating delayed myelination. P1 showed low intensity in T1, and high intensity in T2. As these MRIs were obtained when he was 5 months old, this indicated delayed myelination. P4, P5, and P10 showed low intensity in T1, and high intensity in T2, indicating very severe hypomyelination.

All ten patients having a *PLP1* mutation or duplication showed nystagmus in early infancy, dysmyelination revealed by MRI, and auditory brain response (ABR) abnormalities (Table 1). This triad of presenting symptoms should be the clue to get a clinical diagnosis of PMD. Regarding the radiological findings of the patients, all the provided MRI findings showed dysmyelination with varying degrees (Fig. 4). P4 with the most severe phenotype of PMD showed very low intensity white matter in the T1-weighted imaging, whereas P2 with the mildest form of PMD showed only mildly affected incomplete myelination in T2-weighted imaging. These MRI findings are well-correlated with the clinical severity.

All five patients with nucleotide alterations in *PLP1* displayed the very severe connatal type PMD, whereas P2 whose genome contained a small duplication including *PLP1* showed the milder classical type PMD. These results agree with previous reports showing that the phe-

notypes of patients with genomic duplications are generally milder than those with nucleotide mutations [3]. However, the other four patients with *PLP1* duplications, P1, P3, P4, and P5, displayed the severe connatal form of PMD.

P4 showed a large duplicated region and died when he was 4 years old. On the other hand, P1 contained a very small duplicated segment, similar to P2 in the length, but displayed a very severe phenotype. Thus, it appears that, as suggested by Regis et al., the extent of the duplicated genomic segments does not correlate with clinical severity [35].

According to Lee et al., 65% of patients with *PLP1* duplications have complex rearrangements in nucleotide sequence levels [28]. In this study, we detected *PLP1* duplications by aCGH, and the directions of those duplicated regions were determined by fiber-FISH. However, there is still the possibility that more complicated small rearrangements exist in the duplicated region, particularly in P1. The existence of more complicated rearrangements may explain the reason why there is no correlation between the size of the duplication and the phenotypic severity.

4.6. Differential diagnosis

After genetic evaluation of *PLP1*, the five patients, who did not show any mutations in *PLP1*, were re-evaluated, and one of them was diagnosed as having metachromatic leukodystrophy in the other institution. Another patient showed congenital leukodystrophy with migrating partial seizures in infancy, but no nystagmus and no ABR abnormality, indicating that PMD would be misdiagnosis. Since the other three patients fulfilled the triad described above, the disease-causing mutations might be on the non-coding upstream region of PLP1, or on the other candidate genes for congenital leukodystrophy, including the gap junction protein α12 (GJA12) and others [36,37].

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LETTERS

A Case of Fukuyama Congenital Muscular Dystrophy Associated with Negative Electroretinograms

Fukuyama congenital muscular dystrophy (FCMD) is a congenital dystrophy associated with brain and eye abnormalities.[1] FCMD is an autosomal recessive disorder and occurs only in Japanese. Common ocular findings are optic atrophy, high myopia, cataracts, and weakness of the orbicularis muscles.[1] Abnormal vascular anastomosis and avascularization in the peripheral retina have also been reported. The eyes are only occasionally affected severely, for example, with retinal detachment and microphthalmia.

It was originally believed that patients with FCMD had normal electroretinograms (ERGs),[2] although a slight reduction of the b-wave and reduced ERGs under photopic conditions have been reported.[3,4] We describe an infant with FCMD exhibiting a severe form of ocular phenotype and negative-type ERG under dark-adapted conditions, a finding that to our knowledge has not been reported before.

Case Report

A 1-year-old boy had appeared normal at birth except for a right eye that was slightly microphthalmic. When he was 1 month old, a diagnosis of retinal detachment was made for the right eye (Fig. 1). His left eye was myopic with a tigroid appearance over the entire retina with pallor of the optic disc. The retinal vessels were tortuous, and the temporal peripheral retina was avascularized in both eyes. A scleral encircling buckle and subsequent vitrectomies were used to repair the retinal detachment in the right eye, but the retina remained detached. Before the surgery, the level of creatinine kinase was elevated (3634 IU/l), and the pediatrician suspected congenital muscular dystrophy despite the lack of any distinctive signs or a family history of muscular dystrophy.

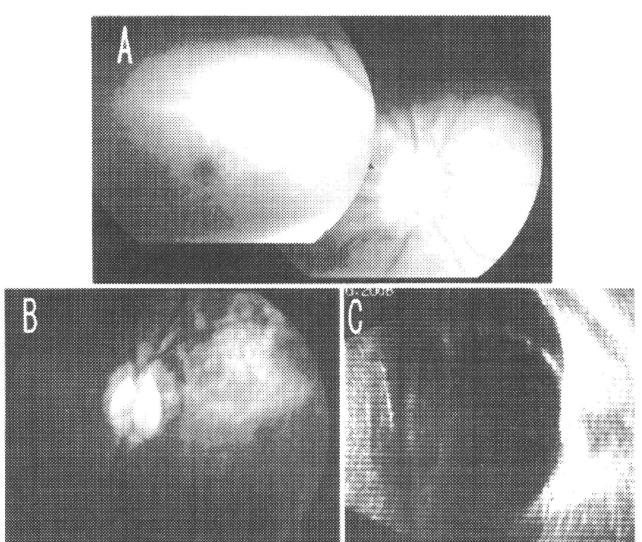

Figure 1A–C. Fundus photographs of our patient with Fukuyama congenital muscular dystrophy (FCMD). **A** Fundus photograph of the right eye showing a temporal retinal detachment involving the macula. **B** Fundus photograph of the left eye showing myopic tigroid appearance with pallor of the optic disc. **C** Ultrasonography showing the retinal detachment in the right eye.

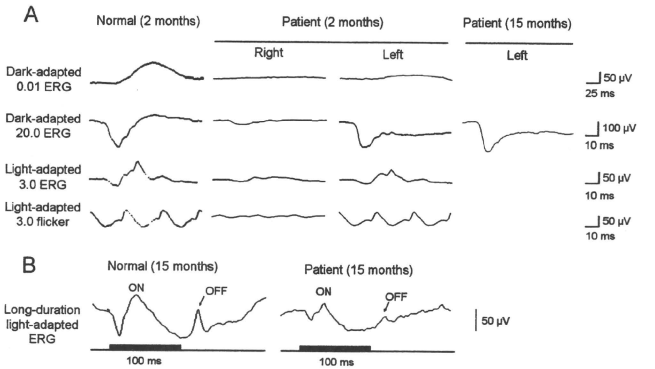

Figure 2A, B. Results of full-field electroretinograms (*ERGs*) using the International Society for Clinical Electrophysiology of Vision-recommended protocol recorded from our patient with FCMD. **A** ERGs recorded in a normal subject at age 2 months (left column), in our patient at 2 months (middle column), and in our patient at age 15 months. The amplitudes of the single-flash cone ERG and 30-Hz flicker ERGs were reduced but better preserved than the rod responses. Note that the maximum rod–cone response (dark-adapted, 20.0 ERG) of our patient showed a "negative" ERG waveform. **B** Light-adapted ERGs elicited by long-duration stimuli in a normal subject at age 15 months (*left*) and in our patient at age 15 months (*right*). Both the ON- and OFF-responses are equally reduced in our patient.

At 5 months, the patient was noted to have muscular weakness and was diagnosed with FCMD. The parents requested genetic testing to confirm the diagnosis. The patient was found to have compound heterozygous mutations in the *FKTN* gene; an insertion mutation in the 3′ noncoding region, and a point mutation, 250C- > T (R47X), in exon 3. The parents were found to be asymptomatic carriers.

During the first vitrectomy under general anesthesia, full-field ERGs were recorded using International Society for Clinical Electrophysiology of Vision-recommended standards (Fig. 2). Because of the retinal detachment, all ERG components in his right eye were severely attenuated. In his left eye, the rod response was decreased to about one-fifth of normal. The a-wave amplitude of the mixed rod–cone ERG was within normal limits, but the b-wave amplitude was smaller than the a-wave amplitude, indicating a negative-type ERG.

Comments

A negative ERG recorded under dark-adapted conditions has not been reported in patients with FCMD and therefore may be a new indication of FCMD. A compound heterozygous mutation in the *FKTN* gene, which occurs infrequently, is most likely the cause of the clinically severe phenotype. This negative-type ERG may be attributable to this less frequent genotype. A similar selective reduction of the b wave was described in patients with muscular dystrophies associated with changes in dystroglycan and dystrophin. The origin of the selective reduction is believed to be a disturbed neurotransmission from the photoreceptors to the ON-bipolar cells.[2]

To determine whether this negative-type ERG was caused by selective impairment of the postsynaptic ON-pathway, we also recorded light-adapted ERGs elicited by long-duration stimuli when our patient was 15 months old. We found that both the ON- and OFF-responses were equally reduced (Fig. 2B). This result is not in accord with the idea that the ON-bipolar cells are selectively disturbed, but an alternative possibility remains that the Müller cells or other neural elements are responsible for the reduction of the b wave because they are also believed to be involved in the generation of the b wave of ERGs.[5]

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Keywords: *FKTN*, Fukuyama congenital muscular dystrophy, microphthalmia, negative ERG, retinal detachment

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Case of a Japanese Patient with X-linked Ocular Albinism Associated with the *GPR143* Gene Mutation

Albinism is an inherited disorder characterized by a reduction or absence of melanin in the hair, skin, and eyes. Albinism can be divided into two broad categories: oculocutaneous albinism and ocular albinism.[1] X-linked ocular albinism (XLOA) is characterized by nystagmus, decreased visual acuity, strabismus, fundus hypopigmentation, macular hypoplasia, and iris hypopigmentation with translucency. It is caused by mutations in the G protein-coupled receptor 143 (*GPR143*) gene (OMIM 300808), originally referred to as the *OA1* gene, which is located at Xp22.32.[2] The fundus of female carriers has a mosaic pattern of pigmentation and depigmentation, which helps in diagnosing XLOA.

We report on a Japanese boy with XLOA whose hair and skin appeared to be hypopigmented, causing some of the referring doctors and his parents to be concerned that he was suffering from oculocutaneous albinism. We detected a *GPR143*/*OA1* gene mutation, making this the first report of this mutation in Japan.

Case Report

The patient was a 4-month-old boy who had been born by normal delivery with a birth weight of 3148 g. His parents noticed that both his irides were blue and his eye movements appeared abnormal from birth. They consulted a pediatrician, who suspected oculocutaneous albinism. The patient was referred to us when he was 4 months old.

No family history of albinism was reported. His hair was mostly light brown, and his skin color was fair for a Japanese individual. He showed pendular horizontal nystagmus but could follow a slowly moving target. His refraction was –0.50 D = cyl –2.00 D Ax 180° (OD) and –0.50 D = cyl –1.50 D Ax 180° (OS). Slit-lamp examination showed that both irides were light brown (Fig. 1A, B). Bilateral foveal hypoplasia was present, and the ocular fundus was albinotic (Fig. 1C, D). Because his mother's fundus showed a mosaic pattern in the midperiphery, XLOA was diagnosed (Fig. 1E, F). He was also seen by a pediatrician of the Hamamatsu University School of Medicine, who diagnosed oculocutaneous albinism rather than ocular albinism (Fig. 1G, H). Because of the discrepancy in diagnoses, his parents wanted the diagnosis confirmed so as to know whether his skin needed to be protected from ultraviolet exposure.

After genetic counseling, the parents agreed to a genetic examination of their child and of themselves. The molecular genetics study was approved by the Institutional Review Board for Human Genetics and Genomic Research of Hamamatsu University School of Medicine. Nine exons and the surrounding regions of the *GPR143* gene were amplified by polymerase chain reaction (PCR) and directly sequenced. A splice mutation at the junction between exon 5 and intron 5, c.658+1G>A, was detected in the patient (Fig. 2). A heterozygous mutation was detected in his mother, but not in his father. The polymorphisms c.251–135C>T and c.767+10C>G were also detected in the patient.

Comments

This is the first report of a Japanese XLOA patient with a *GPR143* mutation. Various types of mutations in *GPR143* have been identified in Caucasian and Chinese populations. The splice mutation c.658+1G>A that we described here has been previously reported.[3]

Most Japanese patients with XLOA have brown irides that show no translucency, nonalbinotic fundi with moderate pigmentation, and normal skin and hair color.[4] However, the iris in our patient was light brown and the fundus was albinotic. His hair color was mostly light brown, and his skin color was fair for a Japanese individual. The skin and hair pigmentation in Caucasians with ocular albinism can be in the normal range but is frequently lighter in color than that of their siblings without XLOA. Recently, a Chinese family with XLOA and a *GPR143* mutation was reported to have iris hyperpigmentation.[5] Although the amount of pigment